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13. ABSTRACT *(Maximum 200 words)*

Diurnal patterns of variation in the extracellular concentrations of aspartate, glutamate, and glutamine in the suprachiasmatic nuclei (SCN) of Djungarian hamsters were determined by in vivo microdialysis. In all hamsters with dialysis probes located in or near the SCN (n=5), pronounced diurnal fluctuations in the extracellular concentration of glutamate were observed. Glutamate levels rose gradually during the latter half of the subjective day and through the night to reach peak values at about lights-on. The extracellular concentration of glutamate was significantly elevated ($p < 0.05$) during the latter half of the dark phase relative to the level at midday. Diurnal fluctuations were not consistently observed in the concentrations of aspartate and glutamine in SCN dialysates, nor were significant variations observed for any of the three amino acids in dialysates from probes located outside the SCN (n=3). The nocturnal rise in extracellular glutamate in the SCN was not attenuated by perfusion with 10 μM tetrodotoxin (TTX), although this concentration of TTX completely blocked increases in extracellular glutamate caused by simultaneous perfusion with 30 μM veratridine.

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Microdyalisis; suprachiasmatic nuclei; glutamic acid; diurnal rhythm; Djungarian hamsters

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Daily Profile of the Extracellular Concentration of Glutamate in the Suprachiasmatic Region of the Siberian Hamster (43641)

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Abstract. **Daily profiles of the extracellular concentrations of glutamate (GLU), aspartate, and glutamine in the region of the suprachiasmatic nuclei (SCN) of the Djungarian hamster were examined by *in vivo* microdialysis. Hamsters with a dialysis probe located in or near the SCN exhibited a diurnal variation in the extracellular concentration of GLU. Glutamate levels rose gradually during the latter half of the subjective day and through the night to reach peak levels near the time of lights-on. The extracellular concentration of GLU was significantly elevated ($P < 0.05$) during the latter half of the dark phase relative to the level at midday. The average peak in extracellular GLU was 217% of the midday value ($P < 0.05$; $n = 5$). Diurnal fluctuations were not consistently observed in the concentrations of aspartate in SCN dialysates. The nocturnal rise in extracellular GLU in the SCN was not attenuated by perfusion with 10 μM tetrodotoxin, although this concentration of tetrodotoxin completely blocked increases in extracellular GLU caused by simultaneous perfusion with 30 μM veratridine. Collectively, these results point to a diurnal rhythm in extracellular concentration of GLU in the SCN region which may not reflect diurnal variations in synaptic activity.** [P.S.E.B.M. 1993, Vol 204]

There is substantial evidence that a light-entrainable oscillator located in the suprachiasmatic nuclei (SCN) is responsible for the generation of circadian physiologic and behavioral rhythms in mammals (1, 2). In rodents, bilateral destruction or surgical isolation of the SCN abolishes circadian rhythmicity (3, 4), which is restored after transplantation of fetal SCN tissue into the third ventricle of SCN-lesioned hosts (5–7). These results, together with the observation that rhythmicity is present in cultured SCN explants for several days (8–12), indicate that rhythm generation is intrinsic to the SCN.

Circadian rhythms, which persist under constant environmental conditions, are normally entrained to the external light:dark cycle. The SCN receive potentially entraining photic information by at least two pathways; a monosynaptic projection from retinal ganglion cells (13–15), the retinohypothalamic tract (RHT), and an indirect retinal projection through the ventral lateral geniculate nucleus/intergeniculate leaflet (16, 17), the geniculohypothalamic tract. The RHT projection alone is both necessary and sufficient to support photic entrainment of the circadian oscillator (18, 19).

Excitatory amino acid (EAA) neurotransmission is thought to play an important role in mediating photic entrainment of the SCN oscillator. In the hypothalamic slice preparation, treatment with EAA antagonists blocks the neurophysiologic response of SCN neurons to optic nerve stimulation (20–22). Furthermore, intracerebral administration of EAA antagonists attenuates the phase-shifting effects of light (23, 24), as well as light-induced expression of immediate early genes in the SCN (24–26). However, little is known regarding the regulation of EAA neurotransmission in the SCN. In this regard, information on the temporal dynamics of EAA release in the SCN may contribute significantly to our understanding of the role of EAA neurotrans-

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mission in SCN function. Therefore, the present report describes the daily pattern of fluctuation in the concentrations of two candidate EAA neurotransmitters, GLU and ASP, in the region of the SCN of Djungarian hamsters maintained under an oscillatory light:dark cycle.

Materials and Methods

Animals. A total of 22 adult male Djungarian hamsters (*Phodopus sungorus*) of approximately 90 days of age were used in the present study. The animals were reared in the Department of Biological Sciences vivarium at Kent State University and maintained under a 16:8-hr light:dark cycle. Food and water were available *ad libitum*.

Microdialysis. Microdialysis probes were of a concentric design and were constructed as described previously (27–29) from a 26-gauge stainless steel outer cannula into which was inserted a 36-gauge fused silica inner cannula (Polymicro Technologies Inc., Phoenix, AZ). A 3.0-mm length of nitrocellulose hollow fiber dialysis membrane (Spectrapore; Fisher Scientific, Pittsburgh, PA [o.d. = 0.23 mm; molecular mass cutoff = 6 kDa]) was inserted approximately 2.0 mm into the outer cannula and attached with epoxy resin (Devcon Corp., Danvers, MA). The distal end of the dialysis tubing was sealed with epoxy resin. The length of the dialysis tubing in contact with brain tissue was approximately 0.5 mm. Probe efficiency was determined by immersing dialysis probes in a standard solution of amino acids (Pierce, Rockford, IL) during flow of artificial cerebrospinal fluid (ACSF; 127 m*M* NaCl, 27.5 m*M* $NaHCO_3$, 2.4 m*M* KCl, 0.5 m*M* KH_2PO_4, 1.1 m*M* $CaCl_2$, 0.85 m*M* $MgCl_2$, 0.5 m*M* Na_2SO_4, and 5.9 m*M* glucose; pH 7.5) at a rate of 1–2 μl/min. Probe efficiencies for glutamate (GLU), aspartate (ASP), and glutamine were 5.0 ± 0.4%, 5.3 ± 0.2%, and 5.7 ± 0.1%, respectively. In two initial experiments, commercially available microdialysis probes were employed (Bioanalytical Systems [BAS], West Lafayette, IN; tip size, 0.5 mm × 1.0 mm).

Implantation of the microdialysis probes was performed under Nembutal anesthesia and the animals were treated with Combiotic (7500 units/hamster) after surgery. Probes were permanently affixed to the top of the skull using stainless steel screws and dental acrylic. The brain coordinates for placement of the probe tip in the SCN were (relative to bregma) as follows: AP = −0.015 cm, L = −0.03 cm, H = −0.74 cm. A section of dialysis tubing attached to the distal end of the probe permitted the continuous collection of ACSF perfusate. After surgery, the animal was placed in a deep-welled, circular cage and allowed to recover for at least 2 days. Samples were collected 24 hr after connecting the probe to a liquid swivel (Instech, Plymouth Meeting, PA) that allowed unrestricted movement of the animal. When specified, perfusate solutions were changed using a liquid switch (CMA 110; BAS). Perfusate was delivered to the probe using a calibrated syringe pump (CMA 100; BAS). Animals were perfused for a 2-hr period at the start of each experiment to obtain stable baseline levels of transmitter release. All collections were made under a photoperiod of LD 16:8 hr with lights-on at 0800 hr, and during the dark period, samples were collected under dim red light (<0.4 lux). Throughout the sampling experiments, visual inspection verified that the animals exhibited normal behavior, including eating, drinking, grooming, and a normal pattern of locomotor activity occurring predominantly during the dark period.

For assessment of the daily pattern in extracellular amino acid concentrations, hourly samples were collected continuously over 24 hr at a flow rate of 1 μl/min. In experiments involving the administration of pharmacologic agents, 20-min samples were collected at a perfusion rate of 2 μl/min. Each collection tube contained 0.05 *M* perchloric acid. Immediately after collection, samples were frozen on dry ice and stored under N_2 at −80°C until analyzed. Probe location was verified histologically from 10-μm thick, mounted frozen sections stained with cresyl violet.

Effect of Chemical Depolarization on the Extracellular Concentration of GLU and ASP. To determine whether changes in the concentration of EAA in SCN microdialysates might reflect local changes in EAA neurotransmitter release, we investigated the effect of chemical depolarization on the concentrations of EAA in SCN microdialysates. Chemical depolarization experiments were conducted during the light phase, beginning between 2 and 6 hr after lights-on. Hamsters were perfused with ACSF for 2 hr before sample collection. A constant flow rate of 2 μl/min was maintained throughout the experiment. Consecutive 20-min dialysate samples were collected into microfuge tubes containing 10 μl of 0.05 *M* perchloric acid, frozen immediately on dry ice and stored at −80°C until analysis. After the collection of at least five baseline samples, groups of hamsters (n = 3) were subjected to one of the following perfusion protocols: perfusion for 1 hr with ACSF followed by 1 hr with 150 m*M* KCl in ACSF (KCl substituted for NaCl); perfusion for 1 hr in calcium-free ACSF containing a mixture of calcium channel blockers (cinnarizine, flunarizine, verapamil, and diltiazem, 0.4 m*M* each) followed by 1 hr with 150 m*M* KCl in calcium-free ACSF (KCl substituted for NaCl) containing calcium channel blockers; perfusion for 1 hr with ACSF followed by 1 hr with ACSF containing 30 μM veratridine; or perfusion for 1 hr with ACSF containing 10 μM tetrodotoxin, followed by 1 hr in ACSF containing 30 μM veratridine + 10 μM tetrodotoxin. After drug infusion, each animal was perfused with ACSF for an additional 1–2 hr. The effects of the

drug treatments on the extracellular concentrations of GLU and ASP were ascertained by comparing the average concentrations of GLU and ASP during the 1-hr period before chemical depolarization with the average concentrations during the 1-hr period during chemical depolarization (Fig. 2).

Effect of Tetrodotoxin on Nocturnal Levels of EAA in SCN Microdialysates. To investigate the possibility that the apparent nocturnal rise in extracellular GLU was due to an acute increase in neuronal activity in the vicinity of the microdialysis probe, the effect of the addition of 10 μM tetrodotoxin in the perfusate on the concentration of GLU in SCN microdialysates was determined. Hamsters ($n = 4$) were perfused with ACSF at a flow rate of 2 μl/min. Samples were collected at 20-min intervals beginning at the middle of the dark phase (0400 hr). Beginning at 0600 hr, animals were perfused with ACSF containing 10 μM tetrodotoxin (TTX), followed by perfusion with ACSF for an additional hour. Samples were collected into microfuge tubes containing 10 μl of 0.05 M perchloric acid, frozen immediately on dry ice, and stored at −80°C until analysis. The effect of TTX treatment was ascertained by comparing the average concentration of GLU in dialysates collected during TTX treatment with the average concentration of GLU during the 1-hr period immediately preceding TTX treatment (Fig. 3).

High-Performance Liquid Chromatography Determination of Amino Acids. Amino acids were determined as their *o*-phthalaldehyde derivatives by reverse-phase high-performance liquid chromatography (Waters amino acid analysis system) with fluorometric detection (30, 31). The stationary phase was a 5-μm C-18 column (Waters Associates). The mobile phase consisted of a nonlinear gradient of methanol (0–65%) against 0.1 M sodium phosphate buffer (pH = 7.2) containing 0.2% methanol and 0.2% tetrahydrofuran. Samples were reacted with an equal volume of 0.5% *o*-phthaldialdehyde and 10% methanol in 0.4 M sodium borate buffer (pH 10.4) for 60 sec at room temperature before injection. Amino acid content was evaluated by comparison of peak areas with those for standards obtained under identical conditions.

Drugs and Reagents. Tetrodotoxin, veratridine, calcium channel blockers, amino acid standards, *o*-phthaldialdehyde, and all salts were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol and tetrahydrofuran were obtained as high-performance liquid chromatography grade from Fisher Scientific.

Statistical Analysis. Drug effects were verified statistically using the two-tailed Student's t test for paired observations. Data used to construct diurnal patterns of amino acid release were subjected to analysis of variance with repeated measures and differences between 3-hr means were tested post hoc for significance using the Duncan's multiple range test.

Results

Representative diurnal profiles of the extracellular concentrations of GLU and ASP in the SCN region are shown in Figure 1. The average concentrations of GLU and ASP in SCN microdialysates during the 24-hr period beginning at 1200 hr were 0.62 ± 0.12 and 0.17 ± 0.05 nmol/ml, respectively ($n = 5$). GLU levels were as much as 217% higher during the latter half of the dark phase in comparison to values observed at midday (0.87 ± 0.15 nmol/ml vs 0.40 ± 0.16 nmol/ml, respectively; $P < 0.05$ [Fig. 1B]). The extracellular concentrations of ASP (and glutamine; data not shown) in the SCN did not fluctuate significantly.

Chemical depolarization of neurons in the vicinity of the dialysis probe using ACSF perfusate containing 150 mM KCl (KCl substituted for NaCl) significantly increased ($P < 0.05$; $n = 3$) the concentrations of GLU

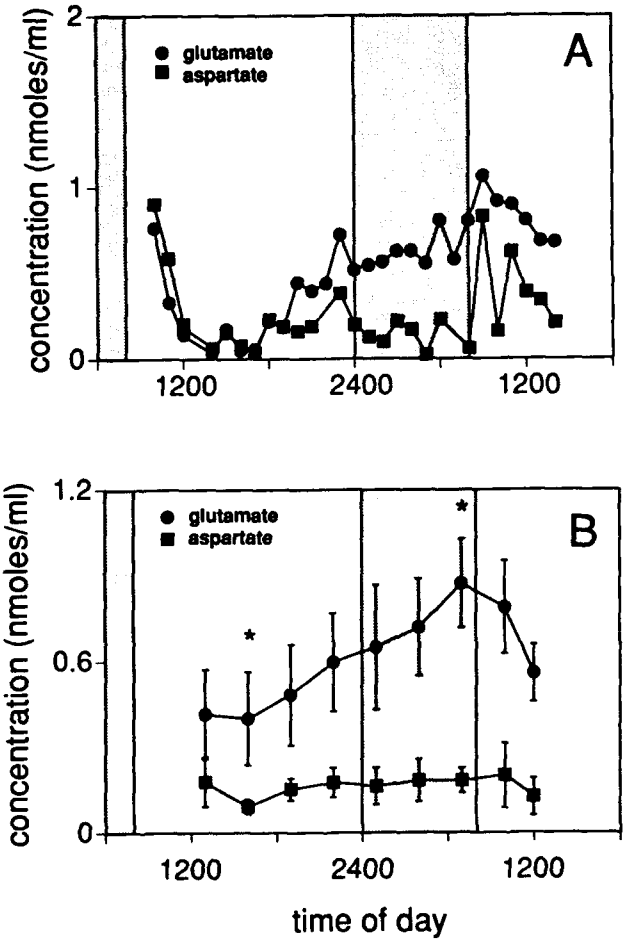
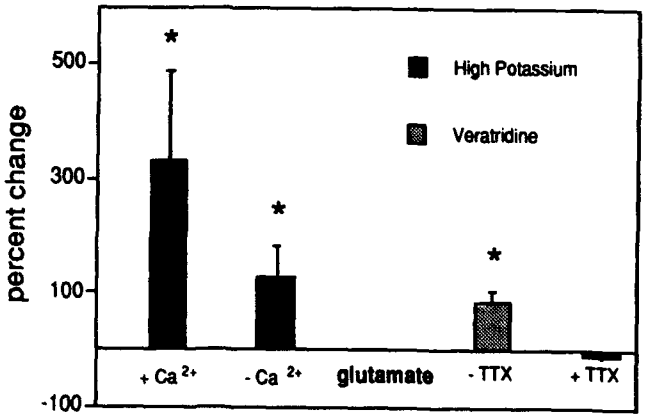


Figure 1. (A) Representative daily profile of the extracellular concentrations of GLU and ASP measured in microdialysates from the SCN region. Data are expressed as nmol/ml of dialysate. Stippling denotes the period of darkness during the 16:8-hr light:dark cycle. (B) Average daily profile of amino acid release from the SCN region. Data represent mean ± SE ($n = 5$) of the average value for the 3-hr intervals centered at the times indicated. The last time point represents the average value for the last 2 hr of perfusion. Asterisks indicate values that differ significantly ($P < 0.05$).

(336 ± 154%) and ASP (127 ± 36%) in SCN microdialysates (Fig. 2). This effect was significantly attenuated by perfusion with calcium-free ACSF containing a mixture of specific calcium channel blockers (cinnarizine, flunarizine, verapamil, and diltiazem, all 0.4 m*M*). Similarly, depolarization with the sodium channel activator, veratridine (30 μM), significantly stimulated the release of GLU (82 ± 18%; $P < 0.01$; $n = 3$), and this effect was blocked by 10 μM TTX (Fig. 2).

Figure 3 shows the effect of local administration of 10 μM TTX to the SCN region during the dark phase (starting at 0400 hr). Perfusion with TTX appeared to cause a transient reduction in the concentration of GLU



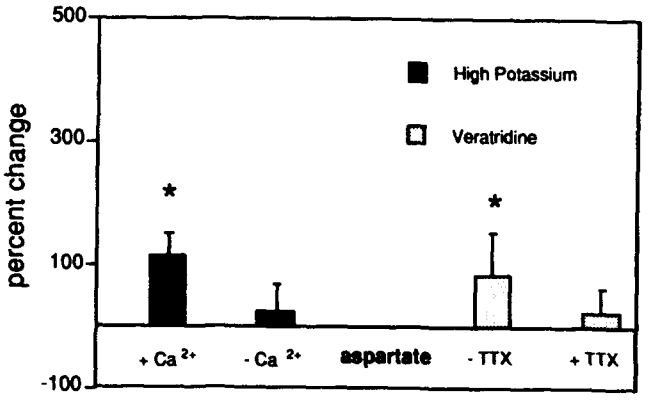


Figure 2. Effects of potassium- or veratridine-induced depolarization on the concentration of GLU (*top*) and ASP (*bottom*) in SCN microdialysates. Data are expressed as the percent change in the concentration of amino acid as described in the Methods. Filled bars represent data obtained during perfusion with ACSF containing 150 m*M* KCl either with 1.1 m*M* calcium ($+Ca^{2+}$) or without added calcium ($-Ca^{2+}$), but in the presence of calcium channel antagonists (cinnarizine, flunarizine, verapamil, and diltiazem, 0.4 m*M* each). Stippled bars denote data obtained during perfusion with ACSF containing 30 μM veratridine in the absence (−TTX) or presence (+TTX) of 10 μM tetrodotoxin. Animals in the $-Ca^{2+}$ and +TTX groups were perfused with calcium channel antagonists or tetrodotoxin for 1 hr before challenge with potassium or veratridine, respectively. Asterisks indicate values that differ significantly ($P < 0.05$) from the average concentration during the 1-hr period before chemical depolarization.

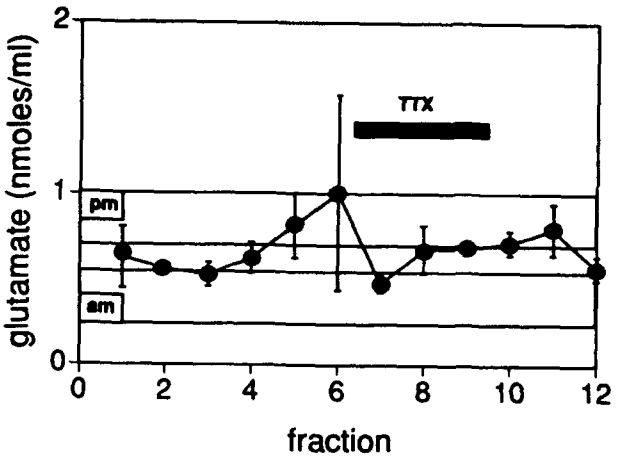


Figure 3. Effect of TTX on the nocturnal increase in the concentration of GLU in SCN dialysates. Data represent the mean ± SE of data collected from four animals with a probe located in or near the SCN. Samples were collected at 20-min intervals beginning at the middle of the dark phase (0400 hr). Animals were perfused with ACSF containing 10 μM TTX for the period denoted by the horizontal bar. For comparison, the stippled areas denote the mean ± SE of the concentration of GLU in SCN microdialysates at 1600 (AM) and 0600 (PM) taken from Figure 1B.

in SCN dialysates. However, a statistical comparison of the average concentration of GLU during the 1-hr period before TTX treatment and the concentration of GLU during TTX treatment showed that TTX failed to significantly reduce extracellular GLU ($P < 0.5$; $n = 4$).

Discussion

Excitatory amino acids appear to play an important role in the regulation of the SCN circadian oscillator, and may participate in the photic entrainment process, possibly as RHT neurotransmitters. Intrahypothalamic injection of GLU or ASP results in stable phase alterations of SCN-driven behavioral rhythms, indicating that the circadian oscillator is responsive to these compounds (32, 33). Furthermore, iontophoretically applied GLU has been shown to alter the excitability of SCN neurons, including a population that is photically responsive (34). In the hypothalamic slice preparation, EAA antagonists have been shown to dose-dependently attenuate both optic nerve stimulation-induced field potentials (20, 22), and excitatory postsynaptic potential recorded in the SCN (21), implicating EAA in RHT neurotransmission. Consistent with this suggestion are several reports showing that local administration of EAA antagonists dose-dependently attenuates light-induced phase alterations of the SCN oscillator and light-induced expression of immediate early genes in the SCN (23–26). However, it is important to point out that the evidence provided by these studies is largely pharmacologic in nature. While substantial evidence indicates that GLU functions as a classical excitatory

neurotransmitter at many synapses in brain, the physiologic release of GLU from retinofugal synapses, including RHT terminals, has not been conclusively demonstrated (35, 36; M. A. Rea, unpublished observations). Although EAA antagonists are effective in blocking the effects of light on the SCN oscillator, EAA agonists fail to mimic photic stimulation (24, 32, 33). In view of available data, it is apparent that the identity of the neurotransmitter(s) responsible for conveying photic information to the SCN oscillator remains unknown.

In the present study, we report temporal patterns in the extracellular concentrations of two EAA neurotransmitter candidates, GLU and ASP, within the SCN region of the Siberian hamster. Extracellular GLU showed a pronounced diurnal pattern, with the lowest levels occurring during the light phase and peak levels occurring during the latter half of the dark phase. Levels appear to fall gradually after lights-on. The concentration of ASP (and glutamine [data not shown]) did not show diurnal fluctuations.

The apparent diurnal rhythm in the extracellular concentration of GLU argues in favor of a role for this neurotransmitter in SCN function; however, this finding reveals little concerning the importance of GLU as a regulator of clock function. The data clearly do not support the hypothesis that RHT terminals are the predominate site of origin of extracellular GLU in the SCN. If this were the case, one would expect that release of GLU from RHT terminals would be higher during the photic phase of the cycle, leading to increased diurnal levels of this amino acid. Thus, while the possibility remains that RHT terminals contribute to the level of extracellular GLU, either during the day or at night, it seems likely that release from elements other than the RHT, such as glutamatergic fibers seen throughout the hypothalamus (37, 38), is primarily responsible for the nocturnal increase.

The possibility that neuronal elements contribute to changes in the extracellular concentration of GLU as detected by microdialysis is supported by the observation that inclusion of membrane depolarizing agents in the microdialysis buffer results in increased GLU in SCN microdialysates (Fig. 3). However, perfusion for 1 hr with 10 μM TTX, a concentration that blocked the rise in extracellular GLU caused by 30 μM veratridine, failed to significantly reduce the nocturnal GLU concentration, suggesting that the nocturnal rise in GLU is not acutely dependent upon action potential-mediated activity. This observation, however, does not eliminate the possibility that the high levels of extracellular GLU observed during the dark phase are of neuronal origin, since modulation of neurotransmitter release by TTX-independent mechanisms has been described (39).

Another possible explanation of the apparent GLU rhythm is that the nocturnal rise in extracellular GLU is not due to increased neuronal activity per se but, rather, is the result of a diurnal variation in GLU uptake by SCN neuroglia. Immunostaining for glial fibrillary acid protein is extremely high in the SCN, indicating the presence of a large population of astrocytes (40). Paulsen and Fonnum (41) have convincingly shown that extracellular concentrations of amino acids, as determined by microdialysis, are directly dependent upon the integrity of glial uptake systems. Furthermore, the suggestion that the rhythm in extracellular GLU may be due to diurnal variations in glial uptake and metabolism is consistent with the inability of TTX to reduce the nocturnal level of GLU (Fig. 3). This hypothesis suggests a potential mechanism by which SCN neuroglia could influence neuronal excitability within the nucleus in a circadian fashion. In this regard, it is noteworthy that chronic TTX administration into the SCN *in vivo*, which blocks both photic input to the nucleus and SCN-driven circadian rhythms (output), does not inhibit the time-keeping function of the nucleus (42).

In conclusion, there is an apparent diurnal variation in the extracellular concentration of GLU in the SCN region of the Siberian hamster, with peak levels occurring 6–8 hr after the nocturnal rise in 5-hydroxyindoleacetic acid in this species (27). The *in vivo* brain microdialysis system as described in the present study is useful for the continuous assessment of neurotransmitter release from discrete brain regions over extended periods of time. Animals undergoing microdialysis exhibit normal behavioral patterns, indicating that this procedure has little aversive effect on the functioning of the SCN or on other neuronal systems that mediate the expression of overt rhythms.

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